POLYMER AFFINITY MATRIX, A METHOD FOR THE PRODUCTION AND USE THEREOF

Technical field

The present invention relates to a polymer affinity matrix for binding one or more substances in a fluid for removing said substance(s) from the fluid and/or decreas-
5   ing the amount or concentration thereof in said fluid with a view to preventing, eliminating, or reducing undesired activation of components or processes in said fluid, to a method for removing said substance(s) from the fluid and/or decreasing the amount or concentration
10   thereof in said fluid, to a method for producing said matrix, to use of said matrix and to a kit comprising said matrix.

The present invention also relates to use a polymer matrix for production of a polymer affinity matrix for
15   removal of one ore more substances from a fluid or decreasing the amount or concentration thereof in said fluid.

Background of the invention

Extracorporeal treatment

20   Extracorporeal treatment of a fluid, such as blood or any other body fluid, requires that the fluid is brought into contact with material in the form of e.g. tubes, lines, beads or membranes. Introduction of such material implies the use of foreign and thus potentially bioincom-
25   patible (e.g. immunologically active or procoagulatory) material. This use of foreign material is associated with activation of the host immune system, e.g. of components in the blood such as lymphocytes, platelets or different types of plasma protein cascades such as proteins in the
30   complement or coagulation cascade. Also, damage to cells such as mechanical or stress-induced damage to e.g. erythrocytes might cause haemolysis and subsequent life-threatening complications to the patient. Treatment of a

2

fluid, such as blood or any body fluid, therefore
requires the use of highly biocompatible material to
avoid undesired activation of components in said fluid,
e.g. blood.

5   Bacterial toxins

Bacterial endo- and exotoxins promote an overwhelming
inflammatory immune reaction in a host due to activation
caused by multiple interactions between blood cells and
soluble proteins in the host and said endotoxin. This
10 immune response is described as an essential feature in
the clinical symptoms of SIRS (systemic inflammatory
response syndrome), sepsis or septic shock. The patho-
genesis is severe and the condition leads to tissue
damage, multiple organ failure, and death induced by
15 sepsis. Searching for new therapeutical drugs and methods
for the treatment of septic patients is important, since
studies of recent therapeutical interventions (Dinarello
et al. in European Cytokine Network 1997; 8:294) show a
failure to protect patients with severe sepsis or septic
20 shock. Also, when manufacturing therapeutical substances
or fluids, care must be taken that the product is non-
pyrogenic, i.e. that the endotoxin concentration is ab-
sent or below accepted limits for excerting its effects.

Endotoxins

25 Endotoxins or "pyrogens" from the outer layers of the
cell membrane of Gram-negative bacteria, e.g. Esherichia
coli, Salmonella typhi, Pseudomonas aeruginosa or Proteus
vulgaris, play an important role in the pathogenesis of
sepsis, septic shock and systemic inflammatory response
30 syndrome (SIRS). Endotoxins, e.g. lipopolysaccharide
(LPS) from E. coli, are composed of a lipid A and a
polysaccharide chain (Zähringer et al. Adv. Carb. Chem.
Biochem., 1994). The molecular structure of LPS is shown
in Fig. 1. The lipid A component is the biologically most
35 active part and mediates the toxic effect of endotoxins
on cells. Lipid A is highly conserved within the

3

different strains of Gram-negative bacteria whereas the
polysaccharide part is much more variable.

Lipid A from E. coli consists of two glucoseamine
moieties, which contain two negatively charged phosphate
5   groups at opposite ends of the two glucoseamines, and six
long-chain highly hydrophobic fatty acid residues which
are linked to the two glucoseamine rings. The variable
polysaccharide chain of the endotoxin is also linked to
one of the glucoseamines.

10   Removal of endotoxins

The removal of endotoxins is difficult and often
includes problems with recovery of or damage to valuable
proteins, i.e. proteins not intended to be removed, or
biocompatibility problems with the blood or body fluid
15   and the means used for removal of the endotoxin. Such a
biocompatibility problem is caused by a mere activation
of the defence mechanisms of the host and involves mul-
tiple cellular activation and release of soluble proteins
such as cytokines and proteins in the complement cascade.
20   The cellular activation and protein release may lead to
severe inflammation, i.e. systemic sepsis or septic
shock, with tissue damage and organ failure as a result.
Blood clotting caused by coagulation is another problem
caused by bioincompatibility. Moreover, the pyrogen might
25   not be completely removed.

Known methods for removing endotoxins include inacti-
vation by the use of heat, acid or alkali (U.S. patents
Nos. 3,644,175, 3,659,027, and 4,070,289). Such methods
often compromise the quality of the final product, i.e.
30   the inactivated detoxified fluid, since the fluid and its
valuable proteins might be denatured or inactivated as
well using this type of processes. Other methods to be
used include adsorption to charcoal, or oxidative decom-
position using an oxidising agent, e.g. potassium per-
35   manganate, aqueous hydrogen peroxide, and sodium hypo-
chlorite.

4

Conventional means for removal of endotoxins

Extracorporeal removal of endotoxins from plasma or whole blood of septic patients is discussed as a potential therapeutic strategy in the treatment of sepsis,

5 septic shock and SIRS. In human plasma there are several binding proteins that play a role in the mediation and inhibition of endotoxin effects on cells, e.g. lipopolysaccharide binding protein (LBP), bactericidal permeability increasing protein (BPI), sCD14, CAP18, and

10 lactoferrin. The peptide antibiotic polymyxin B (produced by Bacillus polymyxa) inhibits the action of endotoxins on cells. The horse shoe crab (Limulus polyphemus) also contains endotoxin-binding proteins and a cell lysate from this species is used for the detection of endotoxins

15 (Limulus amebocyte lysate, LAL, or Limulus test). The binding motifs of such endotoxin binding proteins may be used for extracorporeal treatment of plasma or whole blood of septic patients.

A common characteristic of many endotoxin binding

20 sequences is the presence of alternating positively charged and hydrophobic amino acids in the binding part thereof. It has been shown for one endotoxin binding protein originating from the horse shoe crab that the endotoxin binding sequence forms an amphipatic secondary

25 structure where positively charged residues and hydrophobic residues are located at opposite faces of a loop structure (Hoess et al. EMBO J, 1993). A similar pattern has been proposed for other endotoxin binding sites.

Positively charged polymers such as polyethyleneimine

30 (Mizner et al. Artif. Organs, 1993; Weber et al. ASAIO J, 1995; Petsch et al. J Chromatogr. Biomed. Sci. Appl., 1998) or diethylaminoethyl (DEAE) modified cellulose (Weber et al. A.S.A.I.O. J., 1995) have a certain adsorption capacity for endotoxins, probably based on the

35 interaction of the positive charges with the negatively charged phosphate residues of lipid A. A drawback of polyethyleneimine is the high absorption of heparin and

5

its well-described interaction with platelets which gives
rise to bioincompatibility problems such as coagulation
in an *in* or *ex vivo* application.

   Similarly, positively charged membrane filters are
5   used for purification of infusion fluids. Absorption is
thus dependent on attractive charges and is less specific
and may therefore remove desired valuable proteins as
well.

   Arginine immobilised on sepharose has been suggested
10   for removal of endotoxins from plasma, blood and pharma-
ceutical solutions (EP 0 494 848 and EP 0 333 474).

   WO 92/11847 describes the use of a compound for the
preparation of a medicament to be used orally, intraven-
ously, intramuscularly, intracutaneously or intraperi-
15   toneally for the treatment of endotoxin induced effects
as well as a method for the treatment of endotoxin
induced effects. The compound described in WO 92/11847 is
not immobilised on a solid support.

   Also, hydrophobic polymers (*e.g.* polystyrene, poly-
20   amide, polysulfone, and polyethersulfone) can adsorb
endotoxins in aqueous solutions. This property is used in
ultrafiltration membranes for purification of water and
dialysis/infusion fluid, *i.e.* solutions with low a or no
protein content (Weber *et al.*, Int. J. Artific. Org.,
25   1997). However, the removal of endotoxins from blood or
plasma introduces a competing problem with circulating
plasma proteins (LBP, BPI, sCD14) and of cellular recep-
tors (*e.g.* CD14) to an adsorber matrix due to the plain
hydrophobic absorption which has a low specificity.

30   The use of positively charged or hydrophobic polymers
for the removal of endotoxins shows that the specificity
of the binding and thus also the selectivity for the
removal of the endotoxin in plasma is low. In practice it
has been a challenge to develop an efficient endotoxin
35   adsorbent with both high specificity and high selecti-
vity, still in combination with high biocompatibility.

6

The use of peptide sequences from endotoxin binding proteins has been suggested for therapeutical applications (US patents Nos. 5,639,727 and 5,643,875) by use of e.g. bactericidal/permeability-increasing (BPI) protein
5   products. A drawback of immobilised peptides is the costs of synthesis and the necessity of the peptides to be immobilised without interfering with the binding structure.

Affinity ligands such as histamine, histidine and
10 polymyxin B are effective for the removal of endotoxins though their effectiveness is dependent upon other proteins in the fluid and decreases drastically in the presence of serum proteins like serum albumin or other negatively charged proteins (Anspach and Hilbeck, J.
15 Chromatogr., 1995, Petsch et al. J. Chromatogr., 1997, EP 0 129 786). However, Polymyxin B is toxic to the central nervous system and may cause kidney damage, which is a drawback in marketing approval due to a risk of leakage into the blood of a patient. US 4,771,104 describes an
20 endotoxin detoxifying material comprising a fibrous carrier to which polymyxin B is fixed.

Removal of endotoxins, particularly LPS, from drugs and fluids by use of water-insoluble poly(ε-lysine)(PL) particles was described by Hirayama et al, in Journal of
25 Chromatography B, 271 (1999), 187-195.

Limitations and future perspectives

Efficient endotoxin removal from a protein fluid is dependent on the net charge of the desired valuable protein from which the endotoxin should be removed. The
30 interaction between the endotoxin and its ligand is of both hydrophobic and ionic character, though contribution of each of them depends on the ionic strength and pH of the fluid. Efficient endotoxin removal is also dependent on a high perfusion and biocompatibility of the means for
35 removal of said endotoxin to prevent e.g. cell activation or blood clotting.

7

However, although many means have been developed or suggested for removal of substances such as endotoxins from fluids, e.g. blood, other body fluids or therapeutical fluids, none of the described means is highly bio
5   compatible nor has a high degree of specificity and selectivity against substances to be removed e.g. endotoxins, and at the same time can be easily perfused, e.g. by whole blood or plasma. It is thus desirable to develop highly efficient, biocompatible, and effective
10   methodologies and means for the removal of substances, e.g. endotoxins, from a fluid, and thus making it possible to avoid the problems associated with prior art devices. In this respect, the present invention addresses this need and interest.

15   The need for a novel and efficient method and means for decreasing the concentrations of or removing one or more substances from a fluid, e.g. endotoxins from blood, any other body fluid or therapeutic fluid with a view to preventing, eliminating or reducing undesired activation
20   of components or processes in a fluid is evident from the reasons described above, particularly within the biomedical area. Such a method and means would also be specifically valuable in the treatment of sepsis, septic shock and SIRS by extracorporeal removal of pyrogens or
25   other activating substances from plasma of septic patients.

Further, it is well known to bind a biospecific ligand e.g. an antibody or a peptide to a matrix or substrate and remove by this e.g. pathophysiologically
30   critical substances from the body or blood circulation.

Used known materials are e.g. sepharose (sephadex, pharmacia), polyacrylate or epoxid resins in form of beads or particles.

Known beads are for example made of polymethyl
35   methacrylate e.g. DALI-System (Fresenius) for LDL adsorption; Sepharose: e.g. Rheosorb-System by Plasmaselect for fibrinogen adsorption which uses an

8

immoblized peptide; Therasorb-Immunoadsorption-System to
remove autoantibodies using an immobilized sheep-antibody
which binds human immunoglobulins; Excorim-Protein A
column (and similar systems by other manufacturers) which
5 uses an immobilized bacterial protein to bind immuno-
globulins.

  Known membranes are for example polyamide (MAT/Merck)
and others usually described as affinity membranes.

  Known fabrics are for example Toray polystyrene/poly-
10 ethylene meshes with Polymyxin B ligand for LPS binding.

  All these substrates are modified in case of use of
biological ligands (such as peptides or antibodies.) by
conventional methods of wet chemistry, i.e. first the
corresponding specific ligand is formed or isolated (e.g.
15 by peptide synthesis, by biotechnological methods), then
it is purified and afterwards it is immobilized to a
solid matrix.

  These matrices usually do not allow solid phase
synthesis of a ligand (e.g. a peptide) directly on the
20 matrix, due to chemical incompatibility against the
chemicals (solvents, acids and bases used for cleavage of
protecting groups etc) used in solid phase synthesis.

  The matrices that are suitable for solid phase syn-
thesis (e.g. polystyrene) are not suitable for thera-
25 peutic purposes due to lack of biocompatibility.

  Therefore, a solid phase matrix, which allows solid
phase synthesis as well as contact with blood components
(e.g. plasma or whole blood), would be advantageous for
the following reasons:
30 (1) The development steps to find a suitable ligand
(e.g. an optimised peptide structure) can be performed on
the same solid matrix which can later be used for thera-
peutic application. This means that the necessary bio-
logical test procedures (e.g. measurement of affinity,
35 specificity and binding capacity) can be performed under
conditions very similar to the conditions of the later
therapeutic application. By this the information coming

from the biological test procedures is very reliable and
predictive for the later therapeutic or clinical applica-
tion. This saves time and money and lowers risk of
failure or side effects.

5   (2) A (peptide) ligand can be built-up by solid phase
synthesis in an exactly defined way (with respect to
sequence and geometry). This means also that the linkage
(i.e. the location of the covalent connection) of the
(peptide) ligand to the solid matrix is exactly defined.

10   There are somehow contradicting requirements to such
a matrix with respect to the swelling or wetting
behaviour: for the solid phase synthesis usually non-
aqueous organic (polar, such as dimethylformamide, or
apolar, such as dichloromethane) solvents or mixtures of

15   solvents are used. On the other hand the therapeutic
application must take place in an aqueous environment
(e.g. plasma, blood, therapeutic solutions etc). In both
environments (i.e. aqueous and organic solvents) the
polymer matrix must be easily wettable and swellable:

20   (1) in order to be able to effectively remove or
rinse out unbound (excess) ligand or building blocks
(e.g. protected amino acids) or chemicals (e.g. coupling
agents, such as carbodiimides, deprotection agents, such
as organic acids or bases), and

25   (2) in order to allow the respective toxin to be
removed from the aqueous environment, the matrix must be
wettable and swellable in this environment, too.

          Accordingly, the problem with known polymer matrices
are that you are restricted to the type of regenerating

30   fluid to use in order not to elute the bound active
ligand as such but only the in the material caught sub-
stances to be removed from the fluid, and the ligand is
build up as a defined peptide/molecule or a randomly
polymerised polypeptid/oligomer and has then to be bound

35   to the support material as such - you do not know what
you end up with in the end.

   The advantages with the use according to the inven-
tion are that you can build the ligand directly on the
solid support with the grafted polyethylene glycol and
the solid support with the built ligand could be used
5   directly. This provides for reliability which you do not
get with prior art matrices. Further, it provides for
technological advantages as the biologically active/-
specific ligand is part of the medical device and the
development time is shortened (this could allow or
10   facilitate a kind of individualized treatment).

   During the development the material according to the
invention was not known or expected to work so brilliant-
ly as it turned out to do. The expected drop backs was
e.g. that polymer matrix would swell and plug the flow
15   through the device. Further, the pressure drop when
applying a fluid like whole blood was expected to be too
high. Finally, as a result of the matters above, the per-
fusion of the fluid through the solid phase material was
expected to be insufficient.

20   The polymer matrix according to one of the preferred
embodiments has shown to be excellent for steam sterili-
sation and to thereafter be dried. After drying the
residue of air is easily removed by adding a saline solu-
tion for the polymer matrix to swell again and being
25   ready for use. This leads to short preparation time in
clinic, which is important e.g. in emergencies and in
times of high workload.

   Further, the material shows instant wetting, which is
not the case with most used solid supports used for
30   different types of ligands. This is a very important fea-
ture as a solid phase material for active ligands. Even
further the material is able to be compressed and decom-
pressed within a certain range, and the material is bio-
compatible with respect to complement activation, contact
35   phase activation, cytotoxicity and granulocyte activa-
tion.

Summary of the invention

Therefore, the main object of the present invention is to eliminate the problems associated with the prior art by providing a highly biocompatible, specific, selec-
5   tive and easily perfused polymer affinity matrix for re-
moving and/or decreasing the amounts or concentrations of the above-mentioned undesired substances, e.g. endo-
toxins, in fluids, i.e. a polymer affinity matrix having all the advantages of the prior art and none of the dis-
10   advantages.

This object is achieved according to a first aspect of the present invention, i.e. with a polymer affinity matrix for binding one or more substances in a fluid for removing said substance(s) from the fluid and/or decreas-
15   ing the amount or concentration thereof in said fluid with a view to preventing, eliminating, or reducing unde-
sired activation of components or processes in said fluid, wherein said matrix comprises

a) a solid support
20   b) at least one spacer bound to the solid support, and, coupled to each spacer,

c) a ligand containing at least one binding unit having at least one functional group, said ligand having a defined three-dimensional structure which is complemen-
25   tary as regards charge and/or hydrophobicity/hydrophili-
city to the three-dimensional structure of a binding motif of said substance(s) wherein the polymer affinity matrix has the ability to selectively bind said sub-
stance(s).

30   In a preferred embodiment the present invention re-
lates to a polymer affinity matrix for removal of endo-
toxins to decrease the undesired activation of components or processes in a fluid, wherein the matrix provides a ligand having a three-dimensional structure complementary
35   to the three-dimensional structure of a binding motif of said endotoxin, thereby allowing binding of the endo-
toxin.

12

In the preferred embodiment of the polymer affinity matrix according to the present invention the solid support is a cross-linked polystyrene, the spacer is polyethylene glycol, the at least one binding unit of the ligand is an amino acid, and each functional group is an amino group or guanidino group.

In the preferred embodiment for the removal of endo-toxins to decrease the activation of the fluid, each binding unit in the polymer affinity matrix comprises an amino acid being positively charged at or around the physiological pH of blood, most preferably arginine, lysine, cysteine, or histidine, or another bi- or tri-functional molecule having at least one functional group being positively charged at or around the physiological pH of blood. Such a polymer affinity matrix generates a defined cut-off of about 1×10^{2}-1×10^{6} Daltons and can be used for binding both hydrophobic and/or hydrophilic substances.

In another aspect the present invention relates to a method for removing one or more substances from a fluid and/or reducing the amount or concentration thereof in said fluid with a view to preventing, eliminating or re-ducing undesired activation of components or processes in said fluid, comprising contacting the fluid with the polymer affinity matrix according to the invention for a period of time sufficient to reduce the amount or concen-tration and/or remove said substance(s) of interest, pre-ferably up to 24 hours, most preferably from 1 s to 2 hours. However, the period depends on the flow rate, column size and mode of application, i.e. if the treat-ment is made in vivo, ex vivo or in vitro. Preferably, the amount or concentration of said substance(s) after having been removed or reduced is below the capacity of activating components or processes in blood or prevents activation of components or processes in blood.

13

In a further aspect the invention relates to a method for producing the polymer affinity matrix according to the present invention, comprising the following steps:

a) attaching the spacer to the solid support to obtain a first complex and

b) attaching to said first complex the ligand containing said at least one binding unit with at least one functional group,

or

c) attaching the spacer to the ligand containing said at least one binding unit with at least one functional group to obtain a second complex, and

d) attaching the solid support to said second complex,

or

e) attaching the spacer to the solid support to obtain a first complex, and

f) solid phase synthesis of the ligand on the spacer bound to the solid support,

or

g) building up or synthesizing the spacer from monomers directly on the solid support by grafting, and

h) attaching to said first complex the ligand containing said at least one binding unit with at least one functional group,

or

i) building up or synthesizing the spacer from monomers directly on the solid support by grafting, and

k) solid phase synthesis of the ligand on the spacer bound to the solid support

wherein information about the three-dimensional structure, presence of charges and hydrophobic/hydrophilic regions of the binding motif on the substance(s) to bind is

14

collected from X-ray crystallography, protein
sequencing, protein modelling or hydrophobi-
city and hydrophilicity calculations and the
binding unit is made complementary as regards

5   charge and/or hydrophilicity/hydrophobicity
to the binding motif of said substance(s).

In still a further aspect the present invention
relates to use of the polymer affinity matrix for removal
of one or more substances, preferably endotoxins, from a

10   fluid, or decreasing the amount or concentration thereof
in said fluid, preferably a body fluid or a therapeutic
fluid, most preferably blood.

In still a further aspect, the invention relates to a
kit for removing one or more substances from a fluid

15   and/or decreasing the amount or concentration thereof in
said fluid with a view to preventing, eliminating, or
reducing undesired activation of components or processes
in said fluid, said kit comprising the polymer affinity
matrix, sample tubes, and a device for extra- and/or

20   intracorporeal treatment of said fluid, preferably blood
or serum.

The use of a polymer affinity matrix according to the
present invention will optimise the treatment of fluids,
such as blood, any other body fluid or therapeutic fluids

25   for removal of one or more substances, such as endo-
toxins, and/or reducing the amount or concentration
thereof with a view to preventing, eliminating or reduc-
ing undesired activation of components or processes in
said fluid.

30   Specifically, the use of a highly biocompatible and
perfusable material according to the invention is impor-
tant for prevention of the activation in the fluid during
use of the polymer affinity matrix. This is of particular
importance at extracorporeal removal of endotoxins from

35   plasma or blood of septic patients and is disclosed as a
potential therapeutic strategy in the treatment of
sepsis, septic shock and SIRS. As a further advantage,

the use of the polymer affinity matrix according to the present invention will reduce the treatment time for the patient.

Further advantages and features of the present invention will become more apparent from the following detailed description when taken in conjunction with the drawings and the appended claims.

Brief description of the drawings

Fig. 1 is a schematic view of a lipid A moiety of the LPS molecule with its structural elements.

Figs. 2A and 2B are schematic views of examples of ligands belonging to the tree-like structure (2A) and the comb-like structure (2B) of the polymer affinity matrix according to the present invention.

Fig 2C shows examples of polymer affinity matrixes having a cyclic ligand structure.

Figs 3A-3B show how the complementary structure of the endotoxin is generated by using structural requirements and positive charges, hydrophobic regions and optimal distances in the binding motif.

Fig. 4 describes different ways of producing a polymer affinity matrix according to the present invention.

Fig. 5 shows levels of endotoxin in human plasma after incubation with PS-PEG-beads for different time points (1, 10 and 120 minutes).

Figs 6A-6G show the inhibition of endotoxin induced production of intracellular IL6 in monocytes after treatment of LPS spiked blood with PS-PEG-Arg_8 beads in comparison to beads without the matrix, i.e. reference material (PS-PEG-reference material).

Figs 7A-7D show the biocompatibility profile of PS-PEG-Arg_8 beads measuring white blood cell (WBC) and red blood cell (RBC) numbers, thrombocyte (THR) numbers and hematocrit (HCT).

Fig. 8 shows the lack of TCC (terminal complement complex) formation after use of PS-PEG-Arg_8 beads in

whole blood.

Fig. 9 shows the absence of elastase release after use of PS-PEG-Arg$_8$ beads in whole blood.

Fig. 10. shows the absence of TAT (thrombin-antithrombin III complex) formation after use of PS-PEG-Arg$_8$ beads in whole blood.

Figs 11A-11B show a three-dimensional structure of the ligand containing the binding unit of -Arg$_8$ and -Arg$_4$ optimised for LPS binding.

Fig. 12A shows curve-fitted Langmuir isotherms for PS-PEG-Arg 8 and PS-PEG-Arg 4 related to mass of beads in gram(s).

Fig. 12B shows curve-fitted Langmuir isotherms for PS-PEG-Arg 8 and PS-PEG-Arg 4 related to moles of arginine.

Detailed description of the invention

As indicated above, the present invention relates to a polymer affinity matrix for removal of substances from a fluid, such as blood, any other body fluid or therapeutic fluids, to decrease the activation of components or processes in the fluid, while at the same time having sufficient specificity to avoid adsorption of other valuable substances in the fluid, e.g. physiological components of blood. Said matrix provides a ligand having a structure that is complementary to the structure of a binding motif of the substance to remove or reduce the amount or concentration of, e.g. an endotoxin.

Definitions

In the present context, the term "removing … a substance" is intended to mean preventing, reducing, decreasing, neutralising, inactivating, degrading, modifying, scavenging, binding or concealing a substance, not always intended to mean to a zero amount or concentration of the substance. Moreover, the term "decreasing the amount or concentration of a substance" is intended to mean a reduction or decrease of the amount or concentration of a substance in a fluid enough to prevent or in-

hibit subsequent activation of components in the fluid and/or cellular and/or non-cellular biological mechanisms in the fluid.

With the term "preventing, eliminating, or reducing undesired activation of components or processes" the meaning is to inactivate, inhibit, lower, decrease, reduce, slow down or prevent a certain chemical, biological or biochemical process in the fluid e.g. in blood or tissue cells, e.g. plasma protein cascades such as signal transduction pathways, complement or coagulation processes and/or the activation of components involved in such processes, in a direct or indirect way. A "direct" removal of a substance influences the procedure without any intermediate steps, whereas an "indirect" removal implies removal of a substance being part of a long chain or cascade of reactions, where removal of a substance early will prevent, slow down or inhibit a downstream event of interest. This means that the removal of a substance from e.g. blood may give an inactivated or less activated blood. Also, implied herein is the prevention of activation of the fluid, such as blood.

The term "fluid" is intended to include any fluid, such as any gas or liquid, such as a suspension or a solution, including conventional solutions, e.g. aqueous, or organic solutions, or blood, any body fluid or therapeutical fluid, fluids for life science applications such as fluids in biological, diagnostic or biotechnological applications e.g. buffer solutions, infusion fluids, or dialysis fluids, fluids for nutrition and fluids for industrial use.

The term "body fluid" is intended to include blood, plasma, cerebrospinal fluid, ascites and reinfusion fluids (e.g. after hemoconcentration), blood products obtained from healthy donors, such as plasma, platelet concentrates, erythrocyte concentrates, which are used for transfusions.

The term "therapeutical fluid" is intended to include
peritoneal dialysis fluids, hemodialysis concentrates and
dialysis water, substitution fluids/on line prepared
fluids, infusion fluids, parenteral nutrition fluids,
5   lavage fluids in surgical environment and fluids for
blood component preparation, blood substitutes (e.g. oxy-
gen carriers, modified hemoglobin solutions, artificial
hemoglobin solutions).

The term "fluids for life science application" is
10   intended to include fluids and/or media for cell cultur-
ing tissue engineering, molecular biology (e.g. solutions
of proteins and enzymes used for PCR techniques),
bacteriology, analytics and pharmaceutical preparation.

The term "fluids for nutrition" is intended to in-
15   clude drinking water, fluids for outdoor situations and
reconstitution fluids for food and drinking concentrates
or powders.

The term "solid support" is intended to mean a solid
phase or support or an insoluble matrix whereupon a
20   molecule, e.g. a ligand in the form of a polypeptide, can
be synthesised or coupled with or without a linker or
spacer in-between.

The term "functional group" is intended to mean a
specific atom, or group of atoms, that gives a molecule,
25   i.e. the binding unit in the matrix according to the
present invention, e.g. an amino acid, a fatty acid, a
carbohydrate, a lectin, and a nucleotide, and derivatives
thereof, or combinations thereof, a specific chemical
characteristic or structure, e.g. a positive or a nega-
30   tive charge, hydrophobicity or hydrophilicity, and/or any
other physio-chemical force, e.g. van der Waals forces
and π-π-interactions among aromatic groups or a capacity
to form further bonds, such as hydrogen bonds or covalent
bonds. Examples of functional groups on amino acids are
35   -COOH, -OH, -SH, guanidino, and -NH_2, but may also be a
substituted amino group or any positively charged group
or mixtures thereof.

The term "binding unit" is intended to mean the
molecule mentioned above under the definition of the term
"functional group", wherein said molecule is responsible
for the binding to the substance(s) to be removed and/or
5   reduced, contains at least one functional group and is
included in the ligand bound to a spacer in the polymer
affinity matrix according to the present invention.

The term "binding motif" is intended to mean a three-
dimensional structural and chemical motif, single or
10   repeated, on the substance(s) to be removed.

The term "ligand" means the whole three-dimensional
molecule bound to the matrix via the spacer, i.e. com-
prising at least one binding unit with at least one
functional group. The ligand forms a three-dimensional
15   complementary structure with and binds to the binding
motif on the substance to be removed. Here, "complemen-
tary" is intended to mean a three-dimensional and geo-
metrically defined structure characterised by a capacity
for precise pairing with the three-dimensional complemen-
20   tary structure of a binding motif of the substance to be
removed.

The term "derivatives thereof" used in connection
with a certain compound means one or more compounds
derivatized in such a way that they have the same or
25   essential same function as the compound as such.

Further, isomers of the compounds constituting the
ligand are also intended to be included within the scope
of the invention, provided they show the same or essen-
tially same function as the compounds as such. In the
30   structure formulas, α and ϵ defines, according the nomen-
clature commonly used for amino acids, the amino groups
used for covalent coupling of further molecules.

The term "substance(s)" is intended to mean at least
one component or molecule of interest, soluble or non-
35   soluble, which is intended to be bound to the binding
unit. Examples of substances are toxic substances that
may activate blood cells such as substances derived from

.20

viruses and bacteria, *e.g.* an endotoxin, a blood cell
population, such as a lymphocyte, thrombocyte, granulo-
cyte, dendritic cell, monocyte, endothelial cell, stem
cell, tumour cell; or a blood component or a product from
5   a metabolic activity such as glucose derived molecules or
degradation products thereof, blood clotting proteins,
procoagulatory proteins, inflammatory or proinflammatory
proteins, cytokines, growth factors, hormones, chemo-
kines, uremic toxins, and macrophage migration inhibitory
10   factor. Examples of blood components are infectious
substances causing *e.g.* a contagious disease including
virus particles, prions, or parasites, fungi, pathogen-
loaded blood cells, as well as drugs after overdosing,
pathogenic food additives or other components not origi-
15   nating from the body. Also intended to be included are
pyrogens, particularly bacterial pyrogens, bacterial
exotoxins, products from Gram-positive bacteria, such as
lipoteichonic acid, products from metabolic disturbances,
chronic or acute, as a result of *e.g.* diabetes mellitus,
20   liver disease, uraemia or kidney diseases or inflamma-
tion, as well as adhesion cascades, *e.g.* soluble adhesion
molecules. Preferred substances to be removed are *e.g.*
endotoxins from Gram-negative bacteria, such as LPS, as
well as bacterial DNA or fragments or degradation
25   products thereof, and oligonucleotides, heparin, phos-
phate, blood cells, soluble or cell surface bound pro-
teins.

The term "spacer" is intended to mean a chain-formed
molecule, *e.g.* a polymer, which modifies the solid sup-
30   port in the sense of becoming a part of the solid support
and positions the ligand with the at least one binding
unit containing at least one functional group away from
the solid support and makes it less restricted by steric
hindrance from the solid support and more available to
35   the substance(s) to bind, *e.g.* an endotoxin, cell
population or blood component. The spacer molecule may be
in a linear and/or branched and/or cyclic format. The

spacer length is herein predefined from structural
requirements of the substance(s) to be removed.

The term "distance molecule" is intended to mean
bifunctional molecules within the ligand having the
5   function of creating a structural distance, if desired,
between the binding unit and the trifunctional branching
molecule defined under general formulas I and II below
and/or between the spacer and said trifunctional
branching molecule. The distance molecule may also be in
10   a cyclic format.

The term "pyrogen" and particularly "bacterial pyro-
gen" defines a fever-producing substance, more commonly
of bacterial origin, e.g. an endotoxin.

Here, the term "biocompatibility" is intended to mean
15   the lack of or absence of activating capacity, of e.g.
activation of cells, coagulation, complement cascades or
similar processes, in the sense that the use of the
matrix does not lead to an activation of the immune
system of the patient, or at least if such an activation
20   occurs, it is only to a minor degree. This means that the
use of a biocompatible compound or substance will not
lead to any unwanted or undesired activation of compo-
nents or processes in e.g. blood or other body fluids as
well as mechanical or stress-induced cell death or cell
25   lysis.

The polymer affinity matrix

To create diversity in the polymer affinity matrix
according to the present invention enough to bind and
adsorb a large variety of substances, the ligand may
30   comprise 1-100 functional groups, preferably 1-32 func-
tional groups.

The polymer affinity matrix according to the present
invention may be in the form of a bead, a gel-like
structure, a membrane or part of a membrane, a film, or a
35   net, or a combination thereof.

Specifically, when the matrix is composed according
to the present invention, it will generate a defined cut-

22

off from about 1×10^2 to 1×10^6 Daltons and may bind both hydrophobic and hydrophilic substances without any restriction.

In a preferred embodiment, the polymer affinity
5   matrix is in the form of a PS-PEG bead (e.g. TentaGel®, obtainable from Rapp Polymere Tübingen), using PEG as a spacer. PEG used as a spacer has the advantage of good swelling of the beads in both organic and aqueous solvents and allows, due to its quasi-fluid properties,
10 similar diffusion processes as in a fluid, preferably a diffusion coefficient close to water, i.e. 40%.

The biocompatibility

According to the invention, the polymer affinity matrix should in a preferred form show a high biocompati-
15 bility when used in a specific application, such as extracorporeal treatment of whole blood. A high biocompatibility implies that certain characteristics are intended to be more important than others. For example, no complement activation, measured by early complex formation
20 and quantitation of terminal complement complex (TCC protein) according to Deppisch et al. (Kidney Int. 37:696-706) is important. Also, a low thrombogenicity is desirable for the patient. The degree of thrombogenicity is measured by quantitation of thrombin-antithrombin III
25 complex (TAT) according to Deppisch et al. (Neuphrol. Dial. Transplant Suppl. 3:17-23, 1994) and should not increase during blood or plasma treatment. Still, a steady blood cell number of white and red blood cells before and after treatment is included as well as an
30 absence of cell activation due to contact phase activation. A steady blood cell count intends to mean a low degree of or virtually no damage to blood cells due to mere stress, activation or mechanical damage.

Proteases, such as elastase produced by e.g. granulo-
35 cytes or neutrophiles upon activation, are increasing in patients with bacterial sepsis and septic shock (Heiden et al. Sem. Thromb. Hemost. 1996). Neutrophilic elastase

23

is also suggested as an early and effective marker of
infection (Jensen et al., Scand. J Clin. Lab. Invest,
1996; Groenenveld et al, Cytokine, 1995). Measurements of
elastase may give additional information about biocompa-
5   tibility, and levels should accordingly not change during
blood treatment.

The ligand

       To be able to create a ligand complementary to the
binding motif of the substance to be removed, such as an
10   endotoxin, a three-dimensional structure has to be form-
ed. This may be achieved by the use of e.g. a flexible or
rigid polypeptide structure, which is easy to synthesise
in an optimal way for the required three-dimensional
structure. Such a polymer may be linear or branched, e.g.
15   in a tree- or comb-like structure, or cyclic. Beneficial
to the formation of such three-dimensional structures is
the use of amino acids since they provide flexibility by
nature. The chemistry for coupling such a polymer, e.g. a
polypeptide, is well known within the art. In a preferred
20   embodiment of the present invention such a polymer refers
to a polymer containing more than one amino acid, gene-
rally up to about one hundred, i.e. an oligomer, linked
together by peptide bonds. Examples of linear polymers
are poly- or oligopeptides formed by amide bonds between
25   the alpha-carboxyl and alpha-amino groups of adjacent
residues, and examples of branched polymers are poly- or
oligopeptides formed by amide bonds involving one or more
non-alpha-amino groups.

       As stated above, the ligand molecule may be in a
30   cyclic format. The cyclic structure may be formed by a
covalent coupling between two appropriate functional
groups within the ligand structure, e.g. a disulfide bond
(-S-S-) between two SH groups of cysteine by oxidation (a
common cyclisation reaction also occurring in natural
35   protein structures).

       Figs. 2A and 2B show examples of different branched,
tree-like and comb-like structures, respectively, of the

24

ligand coupled to a spacer bound to a solid support. In
these structures the ligands are basically built up of
lysine residues, and the binding units in each ligand are
arginine residues. The ligand included in the polymer
5   affinity matrix according to the present invention may be
represented by the two following formulas:
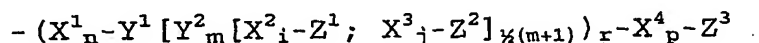
General Formula I:

10       $-X^1{}_n-Y_m[X^2{}_i-Z^1;\ X^3{}_j-Z^2]_{\frac{1}{2}(m+1)}$
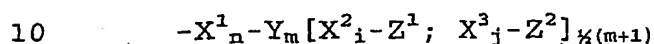
In general Formula I, representing a tree-like structure,
the different symbols have the following meaning:

15   $n = 0$ or 1;
$m = 2^k-1$;
$k = 0$ to 10, wherein if $k = 0$ then $X_2 = X_3$ and $Z_1 = Z_2$;
$i = 0$ or 1; and
$j = 0$ or 1,

20

General Formula II:

    $-(X^1{}_n-Y^1[Y^2{}_m[X^2{}_i-Z^1;\ X^3{}_j-Z^2]_{\frac{1}{2}(m+1)})_r-X^4{}_p-Z^3$

25   In general Formula II, representing a comb-like
structure, the different symbols have the following
meaning:

$n = 0$ or 1;
30   $m = 2^k-1$;
$k = 0-10$, wherein if $k = 0$ then $X_2 = X_3$ and $Z_1 = Z_2$;
$r = 1-100$;
$i = 0$ or 1;
$j = 0$ or 1; and
35   $p = 0$ or 1.

Z^1, Z^2 and Z^3 each represents the binding unit and is each an organic molecule chosen from the group consisting of an amino acid, a peptide, a fatty acid, a carbohydrate, a lectin, and a nucleotide, and derivatives thereof, or

5   combinations thereof, wherein Y, Y^1 and Y^2 each is a trifunctional branching molecule which contains functional groups chosen from the group consisting of amino, hydroxy, aldehyde, isocyanate, isotiocyanate, thiol, maleimido, epoxy, and derivatives thereof, or combinations

10   thereof, and wherein
X^1, X^2, and X^3 each is an optional bifunctional distance molecule containing two functional groups chosen from the group consisting of amino, carboxy, hydroxy, aldehyde, isocyanate, isothiocyanate, thiol, maleimido, epoxy, and

15   derivatives thereof, or combinations thereof.

In Fig 2C examples of polymer affinity matrixes having a cyclic ligand structure are shown. In the structure formulas R means $-Lys_m [Arg]_{(m+1)}$, wherein $m=2^k-1$, and examples wherein $k=0,1$, and 2 are shown.

20   Thus, the at least one binding unit of the ligand in the polymer affinity matrix according to the invention contains at least one functional group, preferably an amino group or an guanidine group or a substituted amino group or at least one of said functional groups.

25   Preferably, the amino acids are chosen for their characteristic of being positively charged at or around physiological pH, specifically of blood ≥ 7.2, i.e. at a pK about ≥ 6.0. Examples of such preferred amino acids are arginine (Arg), lysine (Lys), and histidine (His).

30   Also mixtures of said amino acids may be used in the ligand of the matrix, to create still a further variability.

Most preferably, the ligand comprises arginine as binding unit(s) and constitutes $\leq 3mmol/g$ matrix. In

35   general, the amount of amino acids may be at least about 0.01-5 mmol/g matrix.

26

In the embodiment when the binding unit of a branched
ligand comprises the amino acid arginine, the number of
arginine molecules per ligand is 2-100 arginine mole-
cules, and preferably 4-8 molecules, known as Arg_{4-8}.
5   Still, by using a trifunctional amino acid, i.e. con-
taining three-functional groups, here two amino groups
and one carboxy group, such as lysine, a branched
structure can be created. This approach of using so
called multiple antigenic peptides, MAP, has been intro-
10 duced by Tam et al., and is described in U.S. patent No.
5,229,490. By variation of the number of branches, the
clustering and the distances of the end groups, e.g.
positively charged arginine comprising terminal positive
functional groups, can be varied, as discussed above in
15 connection with Fig. 2.

In a specific embodiment of the invention, where
endotoxins are the substances to be removed by the poly-
mer affinity matrix, the positive charges of the func-
tional groups of the amino acids are held at a distance
20 defined by the distance between individual negatively
charged phosphate groups in an endotoxin as shown in Fig.
3.

As discussed above, the amount and the configuration
of the amino acids create the variability of the com-
25 plementary ligand in the polymer affinity matrix, and may
therefore vary to create sufficient variability for ab-
sorbing a large variety of substances. In another embodi-
ment of the invention the amount of amino acid is at
least about 0.01, 0.1, 1, 2, 3, 4, or 5 mmol/g matrix.
30 Design of the ligand containing the binding unit(s)

To achieve a complementary structure to a substance,
information is collected from procedures known in the art
for studying the relationships regarding structure, pre-
sence of positive charges being held at a certain dis-
35 tance as well as presence of a hydrophobic region in pro-
ximity to the charged groups, e.g. X-ray crystallography,
protein sequencing, protein modelling or hydrophobicity

27

and hydrophilicity calculations as described in Example 1 and shown in Figs 3 and 11.

In the preferred embodiment, the substance to be removed is an endotoxin, such as LPS containing lipid A,

5   and information known to the skilled man in the art about the molecule regarding e.g. structure and distances between charged phosphate groups within the molecule is used to form a ligand with at least one binding unit in the polymer affinity matrix. The polymer forming the

10 ligand is in this embodiment synthesised by amino acids, e.g. arginine and/or lysine. However, other amino acids might be used, such as histidine or cysteine.

In the Arg_{4-8} embodiment of the invention as the complementary part to the binding motif of the substance to

15 remove, e.g. an endotoxin, said arginine as binding units possess the following properties for the removal of the endotoxins as shown in Fig. 3:

   a) presence of positive charges being held at a distance which optimally fits with the distance

20   of the negatively charged phosphate group in lipid A,

   b) presence of a hydrophobic region in proximity to the charged groups allowing hydrophobic interactions with the fatty acid moieties of lipid A,

25   and

   c) a combination of said properties in a) and b) to give a complementary structure to lipid A allowing optimal binding.

The spacer

30 The polymer affinity matrix according to the invention comprises a spacer that is substantially hydrophobic or hydrophilic, modifying the solid support in the sense of becoming a part of the solid support, and has the function of an anchoring part for the ligand comprising

35 the at least one binding unit with at least one functional group.

Preferably, polyethyleneglycol (PEG) is used as
spacer molecule, in a linear or branched configuration at
the preferred average molecular weight of 400-10 000
Daltons and is represented by the formula H-$(OCH_2CH_2)_n$-OH,
5   wherein n is about 2-250. The flexibility of PEG chains
allows a good access of toxin molecules within the three-
dimensional structure of the ligand polymer.

Moreover, in another embodiment of the present inven-
tion the solid support may be provided with two or more
10   spacers of the same or different kind, e.g. polyethylene-
glycols, polypropyleneoxides, polyvinylalcohols,
polyvinylamines, polyglycidoles, or polyethyleneimines,
and derivatives thereof.

Alternatively, the presence of a spacer in the
15   polymer affinity matrix is not required, e.g. when the
three-dimensional structure of the complementary binding
motif of the substance(s) to bind allows the absence of
the spacer for the binding to occur. Thus, according to
this embodiment of the present invention the binding unit
20   is directly bound to the solid support.

The solid support

The solid support should provide variability in poro-
sity and size exclusion characteristics. Also, it should
provide a support for solid phase synthesis for polypep-
25   tide synthesis in the preferred embodiment. Different
solid supports, i.e. polymers, that may be used in the
present invention are described in EP 0 187 391,
WO 99/17120, and the U.S. patent No. 4,908,405. Here, the
solid support is a linear and/or branched biocompatible
30   graft copolymer having a degree of cross-linking of 0.05-
10% and is selected from the group consisting of poly-
vinylalcohols, polyhydroxystyrenes, polymers produced
from chloromethylated polystyrenes and ethylene glycols;
poly- or oligoethylene glycols of the formula
35   H-$(OCH_2CH_2)_n$-OH, where n represents 2 to 250, polyacryl-
ates or polymethacrylates functionalised with hydroxy
groups; and derivatives thereof. In the present invention

the above-mentioned degree of cross-linking may be up to 50%.

The solid support material is selected from a group consisting of, besides the above-mentioned, polystyrene, hydroxyalky-polystyrenes, hydroxyaryl-polystyrenes, hydroxyalky-aryl-polystyrenes, polyhydroxyalkylated polystyrenes, polyhydroxyarylated polystyrenes, iso-cyanatoalkyl-polystyrenes, isocyanatoaryl-polystyrenes, carboxyalkyl-polystyrenes, carboxyaryl-polystyrenes, aminoalkyl-polystyrenes, aminoaryl-polystyrenes, cross-linked polyethyleneglycols, polyacrylates, polymeth-acrylates, cellulose, silica, carbohydrates, latex, cyclo-olefine copolymers, glass, other suitable polymers or combinations thereof. The form of the solid support may be a bead, membrane, particle, e.g. a nanoparticle, net, woven and non-woven fabrics, fibre mat, tube, film, foil or combinations thereof, or cross-linked interpenetrating networks. In a preferred embodiment, the affinity matrix described above consists of cross-linked polystyrene onto which PEG as a spacer is grafted.

A preferred solid support material in the present invention is a bead, having a size sufficient to provide a highly perfusable and biocompatible support, e.g. polystyrene, preferably in the combination with PEG as a spacer to which lysine and/or arginine is attached, which should have no restrictions for hydrophilic or hydro-phobic substances. A list of suitable commercially available beads is shown in Table 1.

Table 1

Commercially available activated beads[a]

_____

Toyo Pearl HW70EC® (TosoHaas)

Toyo Pearl HW65EC® (TosoHaas)

Toyo Pearl AF650M® (TosoHaas)

TentaGel® (Rapp Polymer)

Eupergit C250L® (Röhm)

Eupergit 250® (Röhm)

Fractogel EMD Epoxy® (M) (Merck)

Fractogel EMD Azlactone® (S) (Merck)

Poros EP® (Perkin Elmer-Biosystems)
_____

[a] The commercially available activated beads above can
be conditioned for immobilization of a ligand

Perfusability of the polymer affinity matrix

        A hydrogel like structure is formed when the polymer
affinity matrix is hydrated and, due to the hydration,
5   swells. The swelling capacity is defined as the swelling
due to hydration of the polymer from a dry state to form
the hydrated and gel-like matrix. Such a swelling may be
defined as an increase in weight per volume unit when
hydrated and it may according to the invention be a
10   factor of about 1.5 - 10 times, preferably 3 - 5 times,
when comparing dry and hydrated forms of the polymer
affinity matrix. This swelling capacity allows whole
blood to perfuse completely through the matrix, still
keeping the blood cells and numbers intact.
15   As stated above, in the preferred embodiment, the
polymer affinity matrix as such generates a matrix with a
defined cut off of about $1 \times 10^2 - 1 \times 10^6$ Daltons allowing
blood cells to perfuse and with almost no diffusional
restriction for hydrophilic and/or hydrophobic sub-
20   stances.

Method for removing a substance

The present invention also relates to a method for
removing one or more substances from a fluid and/or
reducing the amount or concentration thereof in said
5   fluid with a view to preventing, eliminating or reducing
undesired activation of components or processes in said
fluid, comprising contacting the fluid with the polymer
affinity matrix according to the present invention for a
period of time sufficient to reduce the amount or concen-
10 tration of and/or remove said substance(s). In a pre-
ferred embodiment using this method, the removal of a
substance, e.g. an endotoxin from blood or any other body
fluid, will thereby directly or indirectly prevent acti-
vation of e.g. blood cells by binding to the undesired
15 substances listed under the definition of "substance(s)"
above. Preferably this may be achieved by contacting the
fluid with the polymer affinity matrix defined above for
a period of up to 24 hours, most preferably from 1 s to 2
hours, giving a less activated or prevented activation of
20 blood and its components.

In a preferred embodiment, the substance to be re-
moved according to the method disclosed in the present
invention is an endotoxin. The present invention also
relates to removal of a defined blood cell type or popu-
25 lation selected from leukocytes, e.g. T and B cells,
monocytes, thrombocytes, granulocytes and/or neutro-
philes. Also intended to be removed by the above describ-
ed polymer matrix are components in an extra- or intra-
cellular signalling transduction pathway selected from
30 the group consisting of glucose and its degrading pro-
ducts, carbonyl compounds, inflammatory and proinflamma-
tory proteins and/or proteins involved in thrombogenesis
or in the complement cascade, bacteria derived constitu-
ents, endotoxins, cells, blood cells, bacteria and virus-
35 es, or pathogen-loaded blood cells, or at least parts or
degradation products thereof, DNA or fragments thereof,
or phosphate, by providing a ligand comprising at least

one binding unit and having a structure which is comple-
mentary to the structure of a binding motif of the sub-
stance.

In this preferred embodiment, an endotoxin, e.g. LPS,
5   is removed to < 50% over two hours during e.g. a static
incubation or during perfusion of a solid phase column.

In this preferred embodiment, the endotoxin is re-
moved during a defined time period of about 1 second to
about and including 2 hours to an amount or concentration
10   inactivating the blood or preventing activation of the
blood, as measured according to an LAL assay, which is
described below. Using the LAL assay is known in the art
and will provide information about the endotoxin levels.
The method according to the invention, which is fast and
15   efficient, will yield levels of the endotoxin according
to the LAL assay, within the above mentioned time period
of 1 s-2 h, below the capacity of activating blood, or
only activating blood cells and components to a minor
degree.

20   Method for producing a polymer affinity matrix

The method for producing a polymer affinity matrix
according to the present invention comprises the follow-
ing steps:

a) attaching the spacer to the solid support
25   to obtain a first complex, and
b) attaching to said first complex the ligand
containing said at least one binding unit
with at least one functional group;
or
30   c) attaching a spacer to the ligand contain-
ing said at least one binding unit with at
least one functional group to obtain a
second complex, and
d) attaching the solid support to said second
35   complex;
or

e) attaching the spacer to the solid support
to obtain a first complex, and

f) solid phase synthesis of the ligand on the
spacer bound to the solid support, or

g) building up or synthesizing the spacer
from monomers directly on the solid support
by grafting, and

h) attaching to said first complex the ligand
containing said at least one binding unit
with at least one functional group,
or

i) building up or synthesizing the spacer
from monomers directly on the solid support
by grafting, and

k) solid phase synthesis of the ligand on the
spacer bound to the solid support,
wherein information about the three-dimensional struc-
ture, presence of charges and hydrophobic/hydrophilic
regions of the binding motif on the substance(s) to bind
is collected from X-ray crystallography, protein modell-
ing or hydrophobicity and hydrophilicity calculations and
the ligand containing the binding unit is made complemen-
tary as regards charge and/or hydrophilicity/hydropho-
bicity to the binding motif of said substance(s).

The method described above also includes in one
embodiment the production of a matrix with a spacer mole-
cule comprising a ligand immobilised on a solid support
according to any of the following ways as shown more in
detail in Fig. 4;

for a) and b) above: activation of the solid support,
coupling of the spacer molecule to the solid support,
synthesis of the ligand containing the binding unit, and
site specific coupling of the ligand to the spacer
molecule, or

for c) and d) above: synthesis of the ligand contain-
ing the binding unit, coupling of the spacer molecule to
the ligand, activation of the solid support, and site

specific coupling of the spacer-ligand complex to the
activated solid support, or,

    for e) and f) above: activation of the solid support,
coupling of the spacer molecule to the activated solid
support, and solid phase synthesis of the ligand contain-
ing binding unit on this support.

    The method containing steps a) - f) described above
also includes in a second embodiment the specific steps
of, for a) and b), activation of the spacer, coupling of
the activated spacer to the solid support, and coupling
the ligand to said activated spacer, or,

    for c) and d), synthesis of the ligand, activation of
the spacer, site specific coupling of the ligand to the
activated spacer molecule and coupling of the spacer-
ligand complex to the solid support, or,

    for e) and f), activation of the spacer, coupling of
the activated spacer to the solid support and solid
synthesis of the ligand on the spacer bound to the solid
support.

    As stated above, preferred by the present invention
is the method above, wherein the solid support, the
spacer and the ligand are immobilised by activation of a
solid support, coupling of the spacer molecule, and solid
phase synthesis of the ligand containing the binding unit
directly on the solid support.

Use of the polymer affinity matrix

The present invention also relates to the use of a poly-
mer affinity matrix according to the present invention,
preferably for use for removal of one or more substances,
preferably endotoxins, from a fluid, or decreasing the
amount or concentration thereof in said fluid, preferably
a body fluid or a therapeutic fluid, most preferably
blood, in particular for the production of less activated
blood or prevention of undesired activation of components
or processes in blood. The polymer affinity matrix is
preferably used as a part in an extracorporeal blood
purification process or in contact with blood or a blood

35

stream, e.g. as an implant in the body to contact blood
or any body fluid, e.g. the vascular system, blood
vessels or in the peritoneal cavity.

Kit comprising the polymer affinity matrix

5   In one embodiment the present invention refers to a
kit for removing one or more substances from a fluid
and/or decreasing the amount of or concentration thereof
in said fluid with a view to preventing, eliminating, or
reducing undesired activation of components in said
10   fluid, said kit comprising a polymer affinity matrix
according to the present invention, sample tubes, and a
device for extra- and/or intracorporeal treatment of said
fluid, preferably blood or serum.

Conclusion

15   The present invention provides means for the removal
of substances from a fluid in a highly efficient and
time-saving way. This is achieved by the use of a polymer
affinity matrix according to the invention that is highly
biocompatible and allows for whole blood to flow through
20   due to good swelling capacity. The efficient means are
also due to the generation of a ligand comprising a bind-
ing unit complementary to the binding motif of the sub-
stance to be removed. Therefore, the present invention
may be applied in e.g. extracorporeal blood treatment
25   such as dialysis and transfusion medicine for therapeutic
applications, stem cell therapy and/or therapeutic cell
therapy, diagnostic applications and also in biotechno-
logy, bioengineering, gene technology, food chemistry and
water preparation and/or purification.
30   The present invention also concerns the use of a
polymer matrix for the production of a polymer affinity
matrix for removal of one or more substances from a fluid
or decreasing the amount or concentration thereof in said
fluid, wherein the specific affinity is dependent on any
35   ligand applied on the polymer matrix, wherein the polymer
matrix includes a solid support and a spacer, wherein the
solid support is made of a material selected from the

group consisting of polystyrene, polyvinyl alcohols,
polyhydroxystyrenes, polymers produced from chloro-
methylated polystyrenes or polyacrylates, poly-
methacrylates functionalised with hydroxy groups,
5   hydroxyalkyl-polystyrenes, hydroxyaryl-polystyrenes,
hydroxyalkyl-aryl-polystyrenes, polyhydroxyalkylated
polystyrenes, polyhydroxyarylated polystyrenes,
isocyanatoalkyl-polystyrenes, isocyanatoaryl-
polystyrenes, carboxyalkyl-polystyrenes, carboxyaryl-
10   polystyrenes, aminoalkyl-polystyrenes, aminoaryl-
polystyrenes, polymethacrylates, cross-linked poly-
ethyleneglycols, cellulose, silica, carbohydrates, latex,
cyclo-olefine copolymers, glass or combinations thereof,
preferably a cross-linked polystyrene, and wherein the
15   spacer is selected from the group consisting of poly- or
oligoethylene glycols of the formula $H-(OCH_2CH_2)_n-OH$,
wherein n represents 2-250.

In a preferred embodiment of the invention the solid
support has the form of a bead, gel, membrane, particle,
20   net, woven or non-woven fabric, fibre mat, tube, film,
foil or combinations thereof or cross-linked inter-
penetrating networks.

In another preferred embodiment of the invention the
spacer is a polyethylene glycol (PEG) in a linear and/or
25   branched configuration and has an average molecular
weight of 400-10 000 Daltons, or derivatives thereof.

In another preferred embodiment of the invention the
polymer matrix has a swelling capacity enough to allow
perfusion of plasma or whole blood.

30   In another preferred embodiment of the invention the
swelling capacity of the polymer matrix is about 1.5-20
fold, preferably 2-6 fold, from a dry state to the
hydrated form.

In another preferred embodiment of the invention the
35   polymer matrix has the form of geltype beads.

In another preferred embodiment of the invention

37

said fluid is an aqueous or organic solution, a body
fluid, preferably blood, therapeutic fluids, fluids for
life science applications, preferably buffer solutions,
infusion fluids or dialysis fluids in biological,
5   diagnostic or biotechnological application, blood
products obtained from healthy donors, such as plasma,
platelet concentrates, erythrocyte concentrates,
preferably oxygen carriers, modified hemoglobin solutions
and artificial hemoglobulin solutions, fluids for
10 nutrition and fluids for industrial use.

In another preferred embodiment of the invention the
polymer matrix has a cut-off value of from about 1×10^2
to about 1×10^6 Daltons and binds hydrophobic and/or
hydrophilic substances.

15 In another preferred embodiment of the invention the
solid support is a cross-linked polystyrene, the spacer
is a polyethylene glycol.

The rational development of specific binding
structure applicable in extracorporeal blood treatment
20 requires: (i) a flexible technology for building up
ligands and (ii) basic requirements on biocompatibility,
toxicology and processability. Solid phase peptide
synthesis is applied to obtain well defined ligand
structures assembled on biocompatible polymer substances,
25 which is, according to one of the preferred embodiments,
polystyrene-polyethylene glycol grafted copolymers
specific for biologically substances.

By systematic variation of the geometrical structure
of ligand motifs we could demonstrate that an increase in
30 ligand affinity by a factor of 10 to 100 relative to the
molar concentration of the building blocks. These
investigations were carried out in human serum, i.e. in
the presence of competitive proteins.

The assessment of blood compatibility was done by in
35 vitro assays in human plasma and/or whole blood. The PS-
PEG base material with or without the ligand is bio-
compatible with respect to complement activation, contact

38

phase activation, cytotoxicity and granulocyte activation.

The base polymer structure shows an advantageous biocompatibility profile especially for extracorporeal application. The applied technology for ligand synthesis allows the processing of specific polymer materials without generation of toxic residuals.

EXAMPLES

Example 1

Design of the ligand containing a binding unit for LPS

This example describes, without limiting the invention, the design of a ligand containing a binding unit for the removal of LPS.

Principle

An efficient removal of toxins, e.g. LPS, requires a ligand with a binding unit that is complementary to the binding motif of the substance to be removed. This includes a three-dimensional aspect of the substance to be removed and a precise arrangement of molecules to optimise a biospecific recognition with characteristics like complementary charges, hydrophobicity and hydrophilicity. This, together with appropriate distances of the aforementioned characteristics, will complete the ligand with its binding unit. Also, the total three-dimensional presentation of such a ligand within a polymer matrix should be optimal in space for a high perfusion, ligand presentation and flexibility of the ligand. Still, a high biocompatibility is a prerequisite for in or ex vivo blood purification in patients. In the described matrix, the accessibility is comparable to or even identical to a non-solid phase aqueous solution. The structure of LPS is shown in Fig. 1. The suggested complementary binding to LPS is shown in Figs 3A-D, wherein the phosphate groups and hydrophobic tail of LPS are considered for the formation of a complementary binding motif. The suggested structure is shown for Arg_4 and Arg_8 in Fig. 2 as well as in a three-dimensional format in Fig. 11. The Arg_8 shows

half of the arginine positions in the left handed Figure and half of the arginine positions in the right hand Fig. 11B. Also, the link to the spacer, here to PEG, is shown.

5 *Molecular simulation*

Molecular simulations were further applied to identify and verify the three-dimensional geometric and chemical structure of the described binding site. The simulations were performed on a Silicon Graphics Octane2
10 Workstation, using the Insight II-Package (Molecular Simulations Inc. (MSI), San Diego, CA) and the optimisation algorithms included herein, e.g. AMBER-forcefields. The results of the tree-dimensional arrangements for Arg_4 and Arg_8 are shown in Figs 11A and B, respectively. Here,
15 the illustration shows the principle of the three-dimensional terminal part of the matrix, without the PEG spacer or solid support.

Synthesis of the ligand

The ligand with its binding unit is synthesised
20 according to Fig. 4, wherein three different way are illustrated. A preferred method of the three is solid phase synthesis directly onto the PEG spacer attached to the solid support. The binding unit is used herein in further examples.

25 Example 2

Comparison of branched and linear ligands

This example describes the adsorption of the endotoxin LPS from human plasma. The example also shows a comparison between linear and branched ligands for the
30 adsorption of the endotoxin.

Principle

Beads are incubated with heparinised human plasma for a time period of two hours. The endotoxin levels are determined after two hours of incubation using an LAL
35 assay.

Material

The following beads are used:

40

PS-PEG-LBP 94-108	endotoxin-binding sequence from LBP, linear
PS-PEG-BPI 85-99	endotoxin-binding sequence from BPI, linear
PS-PEG-Arg$_8$	8-fold branched containing arginine
PS-PEG-NH-Ac	acetylated base-material as control
No beads	Control

Procedure

Incubation of beads in heparinised plasma is carried out at 37 °C. Samples are slowly agitated and beads are

5   removed by centrifugation after an incubation period of two hours.

Analysis

An LAL assay is performed to quantitate the levels of endotoxins after the incubation with beads. The assay is

10   performed by an LAL induced chromogenic substance reaction (Chromogenics, Mölndal, Sweden). Levels are calculated according to a standard curve obtained with defined concentrations of endotoxin in heparinised human plasma.

15   *Results*

Using the LAL assay the following endotoxin concen-trations were found after a two-hour incubation period.

	Endotoxin (IU)/ml
PS-PEG-LBP 94-108	10
PS-PEG-BPI 85-99	9
PS-PEG-Arg$_8$	2
PS-PEG-NH-Ac	10
No beads	8
Starting value	10

Discussion

20   This experiment shows that the endotoxin levels could be decreased 5-fold by using the branched, three-dimen-

41

sional matrix onto the PS-PEG-beads. Beads with a linear
ligand were not as efficient and endotoxin levels in
those samples were still at, or near, starting levels of
the endotoxin.

5   Example 3

Kinetics of endotoxin binding

This example shows the kinetics of endotoxin binding
when beads with a three-dimensional matrix, optimised for
LPS binding, are incubated with plasma and analysed at
10 different points of time.

Principle

The kinetics of absorption is dependent on the struc-
ture and the degree of cross-linking of the polymer mat-
rix. Beads are incubated with heparinised human plasma.
15 After 1, 10, and 120 minutes samples are withdrawn. The
endotoxin levels are determined using an LAL assay.

Material

The following beads are used:

PS-PEG-Arg_8	8-fold branched containing arginine
PS-PEG-Arg_4	4-fold branched containing arginine
PS-PEG-Arg	linear containing arginine
PS-PEG-NH-Ac	acetylated base material (-Ref)

20

Procedure

Incubation of beads in heparinised plasma is carried
out at 37° C. Samples are slowly agitated and test
samples are withdrawn after an incubation period of 1,
25 10, 120 minutes. Beads are removed by centrifugation.

Analysis

An LAL assay is performed for the quantification of
endotoxin levels as in Example 2.

Results

42

Fig. 5 shows the results of the endotoxin levels
measured at different points of time. The Figure shows
that a time period of two hours is needed for efficient
removal, *i.e.* yielding 20-30% endotoxin left of the
5   starting levels in the samples. The linear ligand con-
taining arginine (PS-PEG-Arg) is only removing half the
amount of endotoxins, i.e. a level of 60% left after
120 minutes. Similarly, where the reference beads (PS-
PEG-NH-Ac) were added, the amount of endotoxin after
10 incubation with the beads was still unchanged, i.e. at
starting values, after 120 minutes.

Example 4

Influence of the terminal amino acid and branching of the
polymer

15 This experiment shows the influence of the terminal
amino acid and (arginine vs lysine) and the influence of
branching, non-branched v.s. 4-fold v.s. 8-fold branched,
of the ligand.

Principle

20 Beads are incubated with heparinised human plasma
with or without endotoxin. The endotoxin levels are de-
termined using an LAL assay after two hours.

Material

The following beads are used:

25

PS-PEG-Arg$_8$	8-fold branched containing arginine
PS-PEG-Lys$_8$	8-fold branched containing lysine
PS-PEG-Arg$_4$	4-fold branched containing arginine
PS-PEG-Lys$_4$	4-fold branched containing lysine
PS-PEG-NH-Ac	acetylated base material
PS-PEG-Arg	linear containing arginine
No beads	Control

43

Analysis

An LAL assay is performed as in Example 2.

Results

	Endotoxin (IU/ml)
PS-PEG-Arg$_8$	0.4
PS-PEG-Lys$_8$	1.8
PS-PEG-Arg$_4$	0.01
PS-PEG-Lys$_4$	2.7
PS-PEG-NH-Ac	7.8
PS-PEG-Arg	3.5
No beads	11
Before incubation, *i.e.* starting values	13

5 *Discussion*

The results show that a branched ligand is more effi-
cient than linear forms, and that arginine is several-
folded (4-folded and 200-folded for Arg$_8$ and Arg$_4$ respec-
tively) more efficient than lysine in the removal of

10 endotoxins. Also, the PS-PEG-Arg$_4$ is the most efficient
in the removal of endotoxins after two hours.

Example 5

Reduction of endotoxin dependent IL6 induction in CD14$^+$
monocytes

15 This example describes the reduction of endotoxin
dependent IL6 induction in human monocytes by the use of
PS-PEG-Arg$_8$.

Principle

In response to LPS, monocytes start to transcribe and

20 express IL6. The levels of upregulated IL6 can already be
measured after 4 h by intracellular flow cytometry
analysis (FACS).

Material

Human mononuclear cells from whole blood are used.

25 PS-PEG-Arg$_8$ is used for the removal of endotoxin and PS-
PEG-NH-Ac is included as a control. Lipopolysaccaride (E.
coli strain O55:B5 from Biowittaker Co.) is used for

44

stimulation of the MNC *in vitro*. FACS analysis is per-
formed using a FACSCAN Calibur (Beckton Dickinson®)
Procedure

Whole blood is incubated at 37° C (5% CO_2) for 30
5   minutes with 10 or 30 IU/ml LPS. Samples are incubated in
parallel with and without PS-PEG-Arg_8 beads, as well as
control beads PS-PEG-NH-Ac. The cells are fixed in
PermFix (Beckton Dickinson®) and a double staining
performed with anti-CD14-FITC and anti-IL6-PE. 20 000
10   cells are counted per cell sample for FACS analysis.
Monocytes defined as CD14$^+$ cells normally constitute
about 5% of the total cell population.
Analysis

Monocytes are defined as CD14$^+$ cells in the cell sus-
15   pension. Intracellular IL6 (icIL6) is calibrated to an
internal standard and quantitated in the CD14$^+$ cell popu-
lation using FACS analysis after the incubation with 10
or 30 IU/ml LPS for 30 minutes. Calculations are per-
formed using CellQuest Software Package (Beckton
20   Dickinson®).
Results

Using the FACS data and calculations performed there-
from, analysis by a skilled man in the art of the CD14$^+$
monocyte population generates the following data display-
25   ed in Fig. 6. Shown in the graph is a 70% reduction of
the endotoxin levels when using PS-PEG-Arg_8 beads com-
pared to the control beads (PS-PEG-NH-Ac) and samples
with no beads included. The intracellular levels of IL6
are shown in histograms. On the right hand side, a
30   decrease can be seen in IL6 levels after incubation with
PS-PEG-Arg_8 beads. The lower left row shows the absence
of intracellular IL6 in fresh cells.
Discussion

This experiment shows a rapid and complete inhibition
35   of LPS stimulation in a human CD14$^+$ monocyte population
measured by an inhibition of the LPS dependent IL6 upre-
gulation in monocytes.

45

Example 6
Cell counts after whole blood perfusion

    This example describes, without limiting the invention, the permeabilisation of blood cells after perfusion
5   through the polymer affinity matrix on beads.

Principle

    Blood purification requires no damage to or activation of cells. Mechanical damage, especially of erythrocytes, could lead to haemolysis and subsequent life-
10 threatening complications. A hydrogel like polymer matrix according to the invention can surprisingly be perfused by whole blood cells.

Material

    A highly swellable polymer matrix, PS-PEG-Arg$_8$ beads,
15 are used. Columns, ID 20 mm, are filled with 1g of the matrix, absorbing 4-5 ml water and subsequently prerinsed with physiological saline solution before use. The columns are then used for perfusion of whole blood.

Procedure

20   Fresh whole blood with citrate and LPS (30 IU/ml) is perfused through columns using sterile equipment in a laminar flow. LPS-spiked whole blood is perfused at a flow of 5 ml/min at 37 °C. Blood samples were drawn before and after perfusion and analysed. Cells analysed
25 are red blood cells (RBC), hematocrit (HTC) and free hemoglobin, white blood cell (WBC), platelets, and thrombocyte numbers (TRC).

    Cell number is counted pre- and post column perfusion by a Coulter Counter® (Becton Dickinson).

30   Free hemoglobin is measured as a total hemoglobin level after a complete lysis of erythrocytes followed by a photometric quantification at 405 nm. Integrity of erythrocytes after whole blood perfusion is shown by measurements of free hemoglobin in plasma by a photometric
35 quantification at 405 nm.

Results and discussion

46

Cell numbers and HCT are measured and the results are
shown in Figs 7A (thrombocytes), 7B (HCT), 7C (RBC), and
7D (WBC). In addition, no increase in free hemoglobin is
found, i.e. no haemolysis is induced (data not shown).

5   Data from this experiment shows that, after the initial
transient retention time due to an initial dilution
effect, the cell number is stabilising to pre-column
values. The data shows a permeabilisation of the matrix
material of about 100%, since the pre-column cell count

10   is the same as the post-column cell count. Also, the
cells keep intact, as measured by viable cell count and
red blood cell lysis.

Example 7

Biocompatibility features

15   This example describes parts of the biocompatibility
profile of the PS-PEG-Arg$_8$ matrix.

Principle

Biocompatibility or non-compatibility is herein mea-
sured as complement activation, elastase release, and

20   trombogenicity by thrombin-antithrombin III complex
formation.

Material

As in Example 6, cellulosic material is used as a
reference.

25   Method

Elastase is measured in plasma from whole blood
perfusion using a specific enzyme linked immunosorbent
assay (ELISA; Diagnostic Product Co.).

Complement activation is measured by quantitation of

30   terminal complement complex (TCC protein) is measured
according to Deppisch et al. (Kidney Int. 37:696-706).

TAT, the degree of thrombogenicity, is measured
according to Deppisch et al. (Neuphrol. Dial. Transplant
Suppl. 3:17-23, 1994).

35   Results

In Fig. 8, the formation of TCC is shown over time.
Reference beads, PS-PEG-NH-Ac (TG-base or -ref material)

47

shows a 4-5 fold increase in TCC formation. After an
initial rise over 10-15 min. for PS-PEG-Arg$_8$ the TCC
formation stabilises at pre-perfusion levels.

In Fig. 9, the level of elastase is measured over
5   time. Levels are not changing compared to pre-column
values. Measurements over 35 minutes are shown. For the
reference material the elastase levels are increasing 8-9
fold over 35 minutes.

In Fig. 10, the formation of TAT is shown. No in-
10   crease in TAT formation is detected.
Discussion

Biocompatibility features are important in ex vivo
and in vivo blood treatment, *e.g.* dialysis. The described
matrix shows no activation of the complement system, no
15   activation of the coagulation system and no elastase
release compared to the reference material included, *i.e.*
a high biocompatibility. This, together with the high
perfusion capacity of whole blood as shown in Example 6,
shows that the polymer affinity matrix according to the
20   invention is highly suitable for extracorporeal treatment
of blood, whole blood included.

Example 8

Further endotoxin adsorption experiments

Endotoxin adsorption experiments have been performed
25   in order to show the importance of finding a specific
geometrically defined arrangement of functional groups
(i.e. arginine residues) which build up a ligand for
endotoxin binding. It is shown that the endotoxin binding
does not just depend on the amount of arginine (i.e. the
30   positive charges) but that the defined geometrical
arrangement is more important.
Procedure

Human serum (as a representative of human body
fluids such as blood, plasma etc) was spiked with defined
35   amounts of endotoxin (i.e. 3 IU/ml and 10 EU/ml) and in-
cubated with 40 mg of PS-PEG-Beads containing either the
Arg8 arrangement or the Arg4 arrangement as ligand. The

48

two kinds of beads/ligand arrangements contain different
amounts of arginine: Arg4 contains 0.72 mmol/g and Arg8
contains 1.89 mmol/g. The higher arginine content of the
Arg8 beads is a consequence of the additional branching

5   caused by the lysine bifurcations. After 30 min of incu-
bation the free (i.e. not bound to the surface or matrix)
concentration of endotoxin was measured (by LAL test) in
the supernatant serum.
Analysis

10   Table 2 Results of the incubation experiment with human
serum

spiked serum before incubation		0	3	10	EU/ml
serum after 30 min incubation	no beads	0	3	9.1	EU/ml
	PS-PEG-Arg4	0	1.5	5.5	EU/ml
	PS-PEG-Arg8	0	0.2	1.6	EU/ml

These data were analyzed by using the Langmuir
approach which describes ligand-receptor interactions and

15   equilibria at solid surfaces (e.g. protein adsorption)
(Ref: JD Andrade: Principles of protein adsorption. in:
JD Andrade (Ed.) *Surface and Interfacial Aspects of
Biomedical Polymers*. Volume 2, Protein Adsorption, Plenum
Press New York 1985, pp 1-80.). This analysis is done by

20   plotting the amount of toxin (i.e. endotoxin) bound to
the ligand-matrix (calculated from the difference between
amount added and amount left in the supernatant after in-
cubation) divided by the total number of ligands present
at the matrix versus the equilibrium concentration of the

25   toxin (i.e. the endotoxin concentration after incubation)
in the supernatant. The plots are then fitted to the
mathematical expression of the Langmuir isotherm, which
gives the affinity or association constants for the
different matrix-bound ligands towards the toxin. In Figs

30   12A and 12B examples of the Langmuir approach are shown.

49

Two kinds of Langmuir plots were performed, which differ by the way the total number of ligands present at the matrix is defined:

5   (1) neglecting the different arginine content of the beads, i.e. by just taking the mass of beads in grams as a measure of the total number of ligands (Fig. 12A); this approach is justified since in the practical application of the ligand-polymer-matrix, e.g. in an adsorber device 10 to be perfused with a human body fluid such as blood or plasma in an extracorporeal circuit, it is to a certain extent limited by the total mass or volume of beads which has to be filled into the device in order to achieve a therapeutically effective reduction of the concentration 15 of a toxin in the patient. The limitation is e.g. related to pressure drop across the device, non-specific effects on blood components (proteins and cells) or even storage space for the device.

20 (2) taking into account the different arginine contents of the beads, i.e. by taking the mass of beads in grams divided by the arginine load in mmol/gram as a measure of the total number of ligands (Fig. 12B); this approach is justified by the fact that the arginine content is a main 25 cost factor in the manufacturing of the ligand-polymer-matrix; the result describes the efficiency of the ligands from an economical point of view.

The equilibrium parameters for the different Langmuir plots are shown in Table 3.

30

35

Table 3: Equilibrium parameters taken from the fitted
curves

		PS-PEG-Arg8	PS-PEG-Arg4	unit
analysis 1	affinity constant	1.81	0.15	ml/EU
	saturation concentration	253.9	200.7	EU/g
analysis 2	affinity constant	1.81	0.15	ml/EU
	saturation concentration	479.9	168.6	EU/mmol

Results and discussion

5   The affinity constant describes and characteriz-
es how strongly a certain ligand is able to bind a toxin,
independently of the amount of ligand present. Surpris-
ingly, the affinity constant of the Arg8 ligand was more
than 10-fold higher (i.e. by a factor of 12.3) than the
10   affinity constant of the Arg4 ligand.

The saturation concentration describes the maximum
amount of toxin which can be bound by the respective
ligand-matrix, assuming that unlimited amounts of toxins
are available. Surprisingly the saturation concentration
15   of the Arg8 ligand-matrix was more than 2-fold higher
(i.e. by a factor of 2.8) than the saturation concentra-
tion of the Arg4 ligand-matrix. Since according to the
Langmuir adsorption model the saturation concentration is
completely independent of the affinity constant, this can
20   be interpreted in a way that the specific geometrical
arrangement of the Arg 8 ligand influences the accessi-
bility of the ligand-matrix for the toxins, e.g. by
influencing the morphology (e.g. crystal-like vs. fluid-
ic) of the PEG-part of the ligand-matrix.

25   The following example demonstrates the importance
and relevance of the improved affinity of the Arg8
ligand-matrix endotoxin with respect to the amount of
matrix which is necessary to achieve a therapeutically
relevant reduction in the toxin concentration:

51

Endotoxin concentrations in the range of 0.1 to 1 EU/ml
are found in septic patients (e.g. Nakamura et al. Renal
Failure 2000; 22: 225-234). Assuming a plasma concentra-
tion of 0.3 EU/ml at the start of the treatment and a
5   plasma concentration of 0.03 EU/ml after treatment and a
plasma volume of 4000 ml, this means that 1080 EU endo-
toxin have to be bound to the ligand-matrix during treat-
ment. The amount of matrix necessary to bind this amount
of endotoxin can be calculated from the Langmuir expres-
10   sion using the affinity constants and the saturation
concentrations for the respective ligands. The concentra-
tion at the end of the treatment is then the equilibrium
concentration. Using the equilibrium data from Table 3
this calculation shows that for the Arg8 ligand-matrix
15   only 83 g are necessary, whereas for the Arg4 ligand-
matrix 1201 g are necessary to achieve the therapeutic
goal. Since both ligand-matrixes have a similar density
(mass per volume) this means that the volume and size of
an adsorber device will be much smaller for the Arg8
20   ligand, which facilitates perfusion by plasma or blood.